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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12P 19/56, C12N 15/31, 15/53, 15/63, 15/74, 15/76

A3

(11) International Publication Number: **WO 99/55829**

(43) International Publication Date: 4 November 1999 (04.11.99)

(21) International Application Number: PCT/US99/07016

(22) International Filing Date: 22 April 1999 (22.04.99)

(30) Priority Data:
09/065,606 24 April 1998 (24.04.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 09/065,606 (CIP)
Filed on 24 April 1998 (24.04.98)

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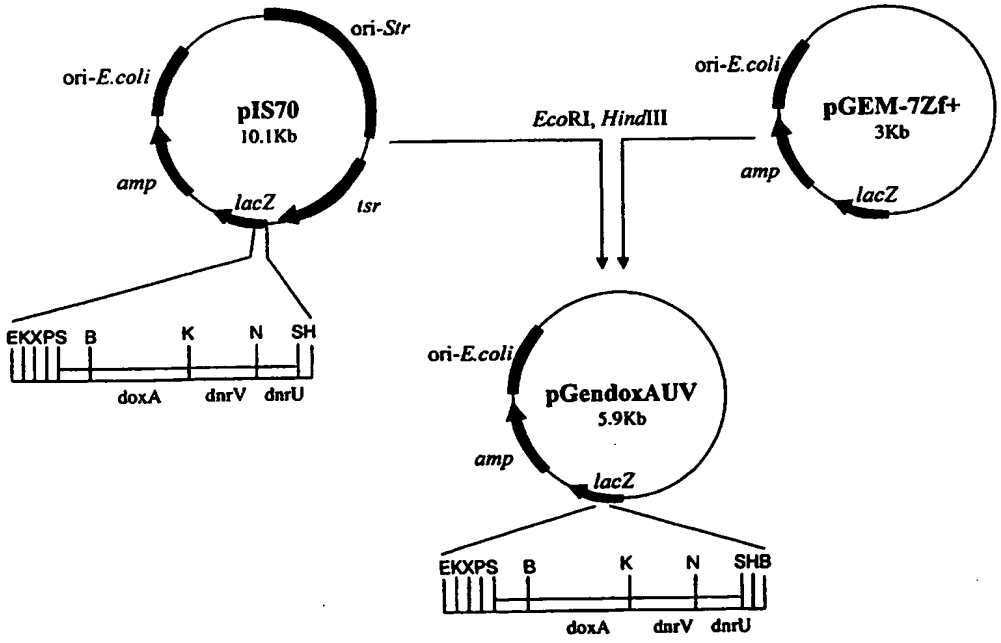
(81) Designated States: CA, CN, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
23 December 1999 (23.12.99)

(54) Title: PROCESS FOR PREPARING DOXORUBICIN



(57) Abstract

The ability to convert daunorubicin into doxorubicin can be improved by transforming a host cell with a recombinant vector comprising a DNA molecule comprising: a DNA region or fragment containing the gene *dox*A encoding daunorubicin 14–hydroxylase and a DNA region or fragment containing one or more gene conferring daunorubicin and doxorubicin resistance.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07016

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 19/56; C12N 15/31, 15/53, 15/63, 15/74, 15/76

US CL :435/78, 252.3, 252.35, 320.1; 536/23.2, 23.7

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/78, 252.3, 252.35, 320.1; 536/23.2, 23.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,695,966 A (INVENTI et al) 09 December 1997, columns 3-8, Figures 1 and 2, and SEQ IDs NOs:1 & 2.	1-19
Y	DICKENS, M. L. Isolation and Characterization of a Gene from *Streptomyces* sp. Strain C5 That Confers the Ability To Convert Daunomycin to Doxorubicin on *Streptomyces lividans* TK24. Journal of Bacteriology. June 1996, Vol. 178, No. 11, pages 3389-3395, especially pages 3390-3394 and Figures 2 and 3.	1-7, 9-12, and 14-19
Y	WO 97/44439 A2 (THE OHIO STATE UNIVERSITY RESEARCH FOUNDATION) 27 November 1997, pages 6-28, Figures 2 and 3 and SEQ IDs NOs:4 and 5.	1-7, 9-12, and 14-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search 07 SEPTEMBER 1999	Date of mailing of the international search report 25 October 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer WILLIAM W. MOORE Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HWANG, K. H. et al. Expression of *Streptomyces peucetius* Genes for Doxorubicin Resistance and Aklavinone 11-Hydroxylase in *Streptomyces galilaeus* ATCC 31133 and Production of a Hybrid Aclacinomycin. Antimicrobial Agents and Chemotherapy. July 1995, Vol. 39, No. 7, pages 1616-1620, especially pages 1617-1619.	1, 2, and 4-19
Y	KAUR, P. Expression and Characterization of DrrA and DrrB Proteins of *Streptomyces peucetius* in *Escherichia coli*: DrrA Is an ATP Binding Protein. Journal of Bacteriology. February 1997, Vol. 179, No. 3, pages 569-575, especially pages 570-574 and Figures 3 and 4.	1, 2, and 4-19
Y	LOMOVSKAYA. N. et al. *The Streptomyces peucetius drrC* Gene Encodes a UvrA-Like Protein Involved in Daunorubicin Resistance and Production. Journal of Bacteriology. June 1996, Vol. 178, No. 11, pages 3238-3245, especially pages 3240-3244 and Figure 2.	4, 6, 10 and 13
Y	WO 97/06266 A1 (ABBOTT LABORATORIES) 20 February 1997, pages 11-13, 15 and 16.	2, 3 and 13
A	US 5,364,781 A (HUTCHINSON et al) 15 November 1994, columns 3-10.	1, 2, 11, 12, 14-17 and 19
A	US 5,652,125 A (SCOTTI et al) 29 July 1997, columns 3-10.	1, 2, 11, 12, 14-17 and 19
A	US 5,665,564 A (CARUSO et al) 09 September 1997, columns 1-6.	1, 4-6 9, and 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07016

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

SEQ ID NO:1, as nucleotide sequence and translated amino acid sequence in N-GeneSeq36, GenEMBL(various), issued U.S. application nucleotide sequences, A-GeneSeq36, PIR60, SwissProt37, SPTREMBL10, issued U.S. application amino acid sequences; STN/Chemical Abstracts; DIALOG/Medline, Biosis, Agricola, Current BioTechnology Abstracts, Derwent Biotechnology Abstracts

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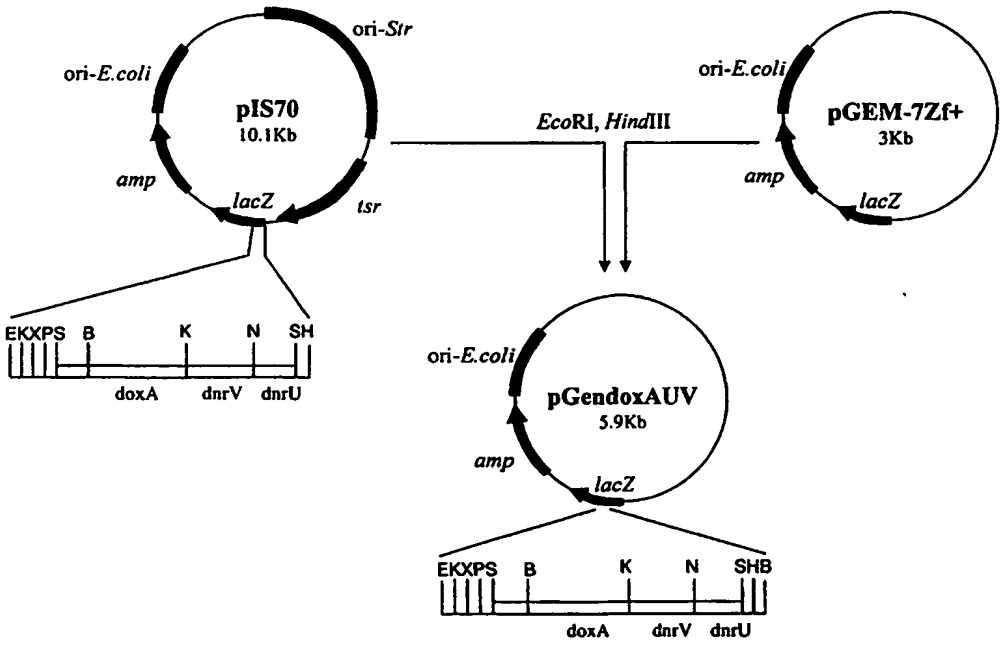

(57) Abstract

The ability to convert daunorubicin into doxorubicin can be improved by transforming a host cell with a recombinant vector comprising a DNA molecule comprising: a DNA region or fragment containing the gene *dox*A encoding daunorubicin 14–hydroxylase and a DNA region or fragment containing one or more gene conferring daunorubicin and doxorubicin resistance.

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Process for Preparing Doxorubicin.

Field of the Invention

The present invention concerns a process for improving daunorubicin to doxorubicin conversion by means of host cells transformed with recombinant vectors comprising DNA encoding a daunorubicin C-14 hydroxylase together with genes conferring resistance to anthracycline antibiotics.

Background of the Invention

Anthracyclines of daunorubicin group such as doxorubicin, carminomycin and aclacinomycin and their synthetic analogs are among the most widely employed agents in antitumoral therapy (F. Arcamone, Doxorubicin, Academic Press New York, 1981, pp. 12; A. Grein, Process Biochem., 16:34, 1981; T. Kaneko, Chimicaoggi May 11, 1988; C. E. Myers et al., "Biochemical mechanism of tumor cell kill" in Anthracycline and Anthracenedione-Based Anti-cancer Agents (Lown, J. W., ed.) Elsevier Amsterdam, pp. 527-569, 1988; J. W. Lown, Pharmac. Ther. 60:185, 1993).

Anthracyclines of the daunorubicin group are naturally occurring compounds produced by various strains of *Streptomyces* (*S.peucetius*, *S.coeruleorubidus*, *S.galilaeus*, *S.griseus*, *S.griseoruber*, *S.insignis*, *S.viridochromogenes*, *S.bifurcus* and *S.sp.* strain *C5*) and by *Actinomyces carminata*. Doxorubicin is mainly produced by strains of *S. peucetius*. In particular daunorubicin and doxorubicin are synthesized in *Streptomyces peucetius* ATCC 29050 and in *S. peucetius subsp. caesius* ATCC 27952. The anthracycline doxorubicin is made by *S.peucetius* 27952 from malonic acid, propionic acid and glucose by the pathway summarized in Grein, Advan. Applied Microbiol. 32:203, 1987 and in Eckart and Wagner, J. Basic Microbiol. 28:137, 1988. Aklavinone (11-deoxy-ϵ-rhodomycinone), ϵ-rhodomycinone, rhodomycin D, carminomycin and daunorubicin are established intermediates in this process. The final step in this pathway involves the C-14 hydroxylation of daunorubicin to doxorubicin.

Genes for daunorubicin biosynthesis have been obtained from *S.peucetius* 29050 and *S.peucetius* 27952 by cloning experiments (Stutzman-Engwall and Hutchinson, Proc.Natl.Acad.Sci.USA 86:3135,1988; Otten et al., J.Bacteriol. 172:3427,1990).The gene encoding the daunorubicin 14-hydroxylase, which converts daunorubicin to doxorubicin has been obtained from *S.peucetius* 29050 and its mutants by cloning experiments and it was overexpressed in the host cells of *Streptomyces* species and *Escherichia coli* as described in WO 96/27014, publication date

Sept.6,1996.

Two genes of the daunorubicin biosynthetic cluster, *drrA* and *drrB*, which confer doxorubicin and daunorubicin resistance to *Streptomyces lividans* have been cloned from *S. peucetius* ATCC 29050 strain (Guilfoile and Hutchinson, Proc.Natl.Acad.Sci.USA 88:8553, 1991) (Accession Number M73758 of Genbank) and from the *S.peucetius* 7600 mutant (EP-0371,112-A and Colombo et al., J.Bacteriol.174:1641,1992). These genes encode two translationally coupled proteins, both of which are required for daunorubicin and doxorubicin resistance in this host. The sequence of the predicted product of one of the two genes is similar to the products of other transport and resistance genes, most notably the P-glycoproteins from mammalian tumor cells. Another gene, *drrC,* which confers resistance to daunorubicin and doxorubicin with a strong sequence similarity to the *Escherichia coli* and *Micrococcus luteus* UvrA proteins involved in excision repair of DNA has been cloned from *S.peucetius* ATCC 29050 (Lomovskaya et al., J.Bacteriol.178:3238, 1996).

Summary of the invention

The present invention provides a process for improving daunorubicin to doxorubicin conversion in host cells by means of recombinant vectors comprising a DNA region or fragment containing the gene *dxrA* encoding daunorubicin 14-hydroxylase together with a DNA region or fragment containing one, two or three genes, selected from the group consisting of *drrA, drrB* and *drrC*, conferring resistance to daunorubicin and doxorubicin. The last three genes confer a high level of resistance in the host cells to doxorubicin, the product of the conversion process, making the process more efficient than the previous one obtained using host cells transformed with the recombinant vectors carrying only the DNA fragment containing the *dxrA* gene, described in WO 96/27014, even when a strong promoter is used.

The DNA of the invention comprises preferably all three of the *drrA, drrB* and *drrC* genes or only the two *drrA* and *drrB* genes.

The DNA may be ligated to a heterologous transcriptional control sequence in the correct fashion or cloned into a vector at the restriction site appropriately located

near a transcriptional control sequence in a vector. Typically, the vector is a plasmid. The recombinant vectors may be used to transform a suitable host cell. The host may be strains of Actinomycetes that do not or do produce anthracyclines, preferably strains of *Streptomyces* .

Brief description of the drawings

Fig. 1 (a-c) illustrate the construction of the plasmid pIS156 described in Example 1. This plasmid was constructed by insertion of the 2.9 kb fragment containing the *doxA* (formerly *dxrA*), the *dnrV* (formerly *dnrORF10*) and the C-terminal part of the *dnrU (ΔdnrU*, formerly *dnrORF9)* genes, obtained from the recombinant plasmid pIS70 (WO 96/27014 and A. Inventi Solari et al., GMBIM '96, P58), under the control of the strong promoter *ermE** (Bibb et al., Molec. Microbiol. 14:533, 1994) into the plasmid pWHM3 (Vara et al., J. Bacteriol. 171:5872, 1989).

In order to better describe the invention, we provide the SEQ.ID. No:1 of 2.867 nt consisting of the *doxA, dnrV* and the C-terminal part of the *dnrU (ΔdnrU)* genes (complementary strand to the coding strand).

Fig. 2 (a-d) illustrate the construction of the plasmid pIS284 described in Example 1. This plasmid contains the 2.9 kb fragment encompassing the *doxA*, the *dnrV* and the C-terminal part of the *dnrU* genes, obtained from the recombinant plasmid pIS70, under the control of the strong promoter *ermE** together with a DNA fragment of 2.3 Kb including the *drrA* and *drrB* resistance genes obtained from the plasmid pWHM603 (P. Guilfoile and C.R. Hutchinson, Proc. Natl. Acad. Sci. USA 88:8553, 1991) subcloned into the plasmid pWHM3.

Fig. 3 (a-c) illustrate the construction of the plasmid pIS287 described in Example 2. Said plasmid was constructed by insertion of the 2.9 kb *BamHI-HindIII* fragment containing the *doxA* formerly, *dxrA), dnrV* (formerly *dnr-ORF10)* and the C-terminal part of the *dnrU (ΔdnrU,* formerly, *dnr-ORF9)* genes, obtained from the recombinant plasmid pIS70 (WO 96/727014), under the control of the strong promoter *ermE** together with the 2.3 kb *XbaI-HindIII* DNA fragment containing the *drrA* and *drrB*

resistance genes and the 3.9 kb *EcoRI-HindIII* fragment containing the *drrC* resistance gene into the plasmid pWHM3.

The maps shown in Figs. 1,2 and 3 do not necessarily provide an exhaustive listing of all restriction sites present in the DNA fragments. However, the reported sites are sufficient for an unambiguous recognition of the DNA segments.

Restriction sites abbreviations: *Ap*, apramycin;*tsr*, thiostrepton, *amp*, ampicillin; B, *Bam*HI; G, *Bgl*II; *N*, *Not*I; *K*, *Kpn*I; *E*, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sph*I; X, *Xba*I, L, *Bgl*I; T, *Sst*I.

Detailed description of the invention.

The present invention provides a DNA molecule in which a DNA region or fragment containing the gene encoding a daunorubicin C-14 hydroxylase is joined to a DNA region or fragment containing one, two or three different genes selected from the group consisting of *drrA, drrB, drrC* genes encoding proteins conferring to the host cells resistance to daunorubicin and doxorubicin.

The DNA region containing the gene encoding a daunorubicin C-14 hydroxylase is preferably the 2.9 kb DNA region obtained from the recombinant plasmid pIS70 described in the patent WO 96/27014 by digestion with *Bam*HI-*Hind*III enzymes. This fragment contains the *doxA* gene, encoding the C-14 hydroxylase. Daunorubicin C-14 hydroxylase converts daunorubicin to doxorubicin. The 2.9 kb DNA fragment also comprises the *dnrV* gene between the *Not*I-*Kpn*I sites and a *Not*I-*Sph*I fragment containing the C-terminal part of the *dnrU* (ΔdnrU) gene.

Preferably, this 2.9 kb DNA fragment encoding a daunorubicin C-14 hydroxylase was ligated to both the 2.3 kb *Xba*I-*Hind*III DNA fragment containing the *drrA* and *drrB* resistance genes obtained from the plasmid pWHM603 and the 3.9 kb *Eco*RI-*Hind*III fragment containing the *drrC* gene obtained from the plasmid pWHM264; in another preferred embodiment, the 2.9 kb DNA fragment is ligated to the 2.3 kb *Xba*I - *Hind*III DNA fragment only.

All the DNA molecules encoding a daunorubicin C-14 hydroxylase described in WO 96/27014 may be employed in the present invention.

In particular the DNA molecule of the present invention may comprise all of the 2.9 kb DNA fragment or only a part of the fragment, at least 1.2 kb in length corresponding to the *Kpn*I-*Bam*HI fragment containing the DNA molecule of *doxA*, encoding a daunorubicin C-14 hydroxylase, which converts daunorubicin to doxorubicin. This DNA molecule consists essentially of the sequence reported in the patent application W0 96/27014, which sequence is referred to as the "*dxrA*" sequence. Also, the deduced amino acid sequence of the daunorubicin C-14 hydroxylase is shown in that patent application.

The DNA molecule of the present invention may comprise at least 2247 nt of the 2.3 kb *Xba*I-*Hin*dIII DNA fragment containing the *drrA* and *drrB* genes encoding proteins conferring to host cells resistance to daunorubicin and doxorubicin.

The DNA molecule of the invention may comprise all or part of the 3.9 kb *Eco*RI-*Hin*dIII fragment containing the *drrC* resistance gene, at least 2.5 kb in length corresponding to the *Sst*I-*Sph*I fragment containing the DNA molecule of *drrC*, encoding a protein conferring to host cells resistance to daunorubicin and doxorubicin.

The present invention also includes DNA comprising genes conferring resistance to doxorubicin and daunorubicin having a sequence at least 80% identical to the sequences of the *drrA* and *drrB* genes (Guilfoile and Hutchinson, Proc.Natl.Acad.Sci.USA 88:8553, 1991) and or *drrC* gene (Lomovskaya et al., J.Bacteriol.178:3238, 1996).

The DNA molecule of the invention may be ligated to a heterologous transcriptional control sequence in the correct fashion or cloned into a vector at a restriction site appropriately located near a transcriptional control sequence in the vector. Preferably the transcription of the different genes may be coordinated by a common strong promoter such as *ermE**(Bibb et al., Molec. Microbiol. 14:533, 1994).

The DNA molecule of the invention may be ligated into any autonomously replicating and/or integrating agent comprising a DNA molecule to which one or more additional DNA segments can be added. Typically, however, the vector is a plasmid. A

preferred plasmid is the high-copy number plasmid pWHM3 or pIJ702 (Katz et al., J. Gen. Microbiol. 129:2703, 1983). Other suitable plasmids are pIJ680 (Hopwood et al., Genetic Manipulation of *Streptomyces*. A laboratory Manual, John Innes Foundation, Norwich, UK,1985) and pWHM601 (Guilfoile and Hutchinson, Proc. Natl. Acad. Sci. USA 88:8553, 1991).

Any suitable technique may be used to insert the DNA into the vector. Insertion can be achieved by ligating the DNA into a linearized vector at an appropriate restriction site. For this, direct combination of sticky or blunt ends, homopolymer tailing, or the use of a linker or adapter molecule may be employed.

The recombinant vector may be used to transform a suitable host cells that do not or do produce anthracyclines.

The host cells may be ones that are daunorubicin or doxorubicin sensitive, i.e., cannot grow in the presence of a certain amount of daunorubicin or doxorubicin, or that are daunorubicin or doxorubicin resistant. In any case the resulting recombinant clones obtained by transformation with the new recombinant vectors of the invention show higher level of resistance to daunorubicin and doxorubicin than the parental host. The level of doxorubicin resistance in recombinant *S. lividans* is much higher than the level observed in anthracycline producing strains *S. peucetius* ATCC 29050 and ATCC 27952.

The host may be a microorganism such as a bacterium. Strains of Actinomycetes, in particular strains of *S. lividans* and other strains of *Streptomyces* species that do not produce anthracyclines may be transformed. *S. lividans* TK 23 is a more suitable host in comparison to the *S. peucetius dnrN* mutant transformed with the recombinant plasmid pIS70 containing the *dxrA* gene used for daunorubicin to doxorubicin bioconversion (WO 96/27014).

The recombinant vectors of the invention may also be used to transform a suitable host cell which produces daunorubicin, in order to enhance the conversion of daunorubicin to doxorubicin.

S. peucetius ATCC 29050 and ATCC27952 strains including their mutants that produce

anthracyclines may therefore be transformed. In particular *S. peucetius* strain WMH1654, a mutant strain obtained from *S.peucetius* ATCC 29050 and deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA, under the accession number ATCC55936 may be used.

Transformants of *Streptomyces* strains are typically obtained by protoplast transformation.

The invention includes processes for improving doxorubicin production by conversion of daunorubicin, which processes comprise a bioconversion process of added daunorubicin into doxorubicin in hosts which do not produce anthracyclines and a fermentation process for producing doxorubicin in hosts which directly produce daunorubicin.

Bioconversion process of daunorubicin to doxorubicin.

This process comprises:

1) culturing the recombinant host cells not producing daunorubicin transformed with the vectors of the invention to which daunorubicin is added and

2) isolating doxorubicin from the culture.

In this process the recombinant strain may be cultured at temperatures from 20°C to 40°C, for example from 24°C to 37°C. The daunorubicin is added to the culture medium from 24 to 96 hours of the growth phase. The culture is preferably carried out with shaking. The duration of the culture in the presence of daunorubicin may be from 12 to 72 hours. The concentration of daunorubicin in the culture may be from 20 to 1000 mcg/ml; for example from 100 to 400 mcg/ml.

Doxorubicin production by fermentation.

This process comprises:

1) culturing recombinant daunorubicin-producing host cells transformed with the vectors of the invention and

2) isolating doxorubicin from the culture.

In this process the recombinant strain may be cultured at temperature from 20°C

to 40°C; for example from 26°C to 34°C. The culture is carried out with shaking. The duration of the culture may be from 72 to 168 hours.

Materials and Methods

Bacterial strains and plasmids: *E. coli* strain DH5α, which is sensitive to ampicillin and apramycin is used for subcloning DNA fragments. The host *S. lividans* TK23 was obtained from D. A. Hopwood (John Innes Institute, Norwich,United Kingdom) and the host *S. peucetius* WMH1654 is a mutant strain obtained from *S.peucetius* ATCC 29050 and has been deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA, under the accession number ATCC55936.

The plasmid cloning vectors are pGem-7Zf(+) and related plasmids (Promega, Madison, WI), pIJ4070 (D. A. Hopwood) and the *E.coli-Streptomyces* shuttle vector pWHM3 (Vara et al., J. Bacteriol. 171:5872, 1989).

Media and buffer: *E. coli* strain DH5α is maintained on LB agar (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). When selecting for transformants, ampicillin or apramycin are added at concentrations of 100 micrograms/ml.

S. lividans TK23 and *S. peucetius* WMH1654 are maintained on R2YE (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and ISP4 (Difco, Detroit, MI) agar media, respectively. When selecting for transformants, the plates are overlayed with soft agar containing thiostrepton at a concentration of 50 micrograms/ml.

Subcloning DNA fragments: DNA samples are digested with appropriate restriction enzymes and separated on agarose gels by standard methods (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). Agarose slices containing DNA fragments of interest are

excised from a gel and the DNA is isolated from these slices using the GENECLEAN device (Bio101, La Jolla, CA) or an equivalent. The isolated DNA fragments are subcloned using standard techniques (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989) into *E. coli* for routine manipulations, and *E. coli-Streptomyces* shuttle vectors or *Streptomyces* vectors for expression experiments.

Transformation of *Streptomyces* species and *E. coli*: Competent cells of *E. coli* are prepared by the calcium chloride method (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989) and transformed by standard techniques (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). *S. lividans* TK23 is grown in liquid R2YE medium (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and harvested after 48 hr. The mycelial pellet is washed twice with 10.3% (wt/vol) sucrose solution and used to prepare protoplasts according to the method outlined in the Hopwood manual (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985). The protoplast pellet is suspended in about 300 microlitres of P buffer (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and 50 microlitres aliquot of this suspension is used for each transformation. Protoplasts are transformed with plasmid DNA according to the small scale transformation method of Hopwood et al. (*Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985), Stutzman-Engwall and Hutchinson (Proc. Natl. Acad. Sci. USA. 86:3135, 1988) or Otten et al. (J. Bacteriol. 172: 3427, 1990). After 17 hr of regeneration on R2YE medium at 30°C, the plates are overlayed with 200 micrograms/ml of thiostrepton and allowed to grow at 30°C until sporulated.

Evaluation of daunorubicin and doxorubicin resistance level: The level of resistance is expressed as Minimal Inhibitory Concentration (MIC) and is determined by the standard two-fold dilution method using R2YE medium. The strains are cultured in slants of R2YE medium and incubated at 28°C for 8-10 days. Recombinant strains are grown in the same medium added with 20 micrograms/ml of thiostrepton. Bacterial cultures containing approximately 10^{6}-10^{7} viable cells/ml are prepared from cultures grown at 28°C at 280 rpm for 48 hours in Tryptic Soy Broth (Difco). The cultures are homogenized by glass beads. One loopful of the homogenized cultures is inoculated on the agar plates containing different concentrations of daunorubicin and doxorubicin from 0.39 to 800 micrograms/ml. The agar plates are incubated at 30°C for 7 days and the MICs are determined as the lowest concentrations that prevent visible growth.

Daunorubicin to Doxorubicin bioconversion: *S. lividans* TK23 transformants harboring a plasmid of the invention are inoculated into 25 ml of liquid R2YE medium with 40 micrograms/ml of thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks and incubated on a rotary shaker at 280 rpm at 30 C°. After 2 days of growth, 2.5 ml of this culture are transferred to 25 ml of APM production medium: ((g/l) glucose (60), yeast extract (8), malt extract (20), NaCl (2), 3-(morpholino)propanesulfonic acid (MOPS sodium salt) (15), $MgSO_4$.$7H_2O$ (0.2), $FeSO_4$.$7H_2O$ (0.01), $ZnSO_4$.$7H_2O$ (0.01), supplemented with 20 micrograms/ml of thiostrepton. 400 micrograms/ml of daunorubicin are added at 48 hr.of the growth phase. Cultures are grown in 300 ml Erlenmeyer flasks and incubated on a rotary shaker at 280 rpm at 30 C° for 72 hr. Each culture is acidified with 25 milligrams/ml of oxalic acid and after incubation at 30°C on a rotary shaker at 280 rpm for 30 min. is extracted with an equal volume of acetonitrile:methanol (1:1) at 30°C and 300 rpm for 2 hr. The extract is filtered and the filtrate is analyzed by reversed-phase high pressure liquid chromatography (RP-HPLC). RP-HPLC is performed by using a Vydac C_{18} column (4.6 x 250 millimeters; 5

micrometers particle size) at a flow rate of 0.385 ml/min. Mobile phase A is 0.2% trifluoroacetic acid (TFA, from Pierce Chemical Co.) in H_2O and mobile phase B is 0.078% TFA in acetonitrile (from J.T.Baker Chemical Co.). Elution is performed with a linear gradient from 20 to 60% phase B in phase A in 33 minutes and monitored with a diode array detector set at 488 nm (bandwidth 12 micrometers). Daunorubicin and doxorubicin (10 micrograms/ml in methanol) are used as external standards to quantitate the amount of these metabolites isolated from the cultures.

Doxorubicin production: The *S. peucetius* WMH1654 mutant is transformed with a plasmid of the invention. Transformants are inoculated into 25 ml of R2YE medium supplemented with 20 micrograms/ml thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks on a rotary shaker at 280 rpm at 30°C. After 2 days of growth, 2.5 ml of this culture are transferred to 25 ml of APM medium supplemented with 20 micrograms/ml thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks on a rotary shaker at 280 rpm at 28°C for 96 - 120 hours. Each culture is acidified with 25 milligrams/ml of oxalic acid and, after 45 min. incubation at 30°C on a rotary shaker at 280 rpm, is extracted with an equal volume of acetonitrile:methanol (1:1) at 30°C and 300 rpm for 2 hr. The extract is filtered and the filtrate is analyzed by RP-HPLC following the same method used to analyze the bioconversion products.

Example 1

Example 1 (Fig. 1 (a-c) and Fig. 2 (a-d).

In order to remove a non-essential region, the plasmid pIS70 (WO96/27014) is before digested *EcoRI-HindIII* and the 3.5 kb fragment is subcloned into the same sites of the multiple cloning site sequence of the plasmid pGEM-7Zf (+) (Promega, Madison-WI USA) to obtain another *BamHI* restriction site. The new plasmid pGendoxAUV was *BamHI* digested and the fragment, now reduced to 2.9 kb, was transferred into the

plasmid pIJ4070 (from the John Innes Institute, Norwich, UK) under the control of strong promoter *ermE**. This new plasmid, named p7doxAUV, was digested *BglII* and the fragment inserted into the plasmid pWHM3 (J.Vara et al., J. Bacteriol. 171:5872-5881, 1989) to obtain the plasmid pIS156 (fig. 1c).

The 2.3 kb *BglI* fragment containing the *drrA* and *drrB* resistance genes is transferred after blunt ending from the plasmid pWHM603 into the *SmaI* site of the plasmid pBluescript II SK + (Stratagene) to obtain the plasmid pdrrAB and an *XbaI-HindIII* fragment is transferred from pdrrAB into the vector pIJ4070 to obtain pIS278. Afterwards, pIS278 is digested with *EcoRI-XbaI* and inserted into the *EcoRI-XbaI* plasmid pWHM3 to obtain the plasmid pIS281. This plasmid is digested with *XbaI* and the *XbaI* fragment of plasmid pIS156 is inserted to obtain the plasmid pIS284.

Example 2

Construction of the plasmid pIS287 (Fig.3 (a-c)): The *drrC* resistance gene contained in the plasmid pWHM264 is excised by *Eco*RI-*Hin*dIII digestion and inserted into the plasmid pIJ4070 to obtain the plasmid pIS282. From this plasmid, the *drrC* resistance gene is transferred as a *Bgl*II fragment to pIS252 (this plasmid is a modified form of pWHM3 containing an extra *Bgl*II site close to the *Eco*RI site) to obtain the plasmid pIS285. pIS285 is *Eco*RI digested and ligated with the 5.5 kb DNA fragment excised from plasmid pIS284 to obtain the plasmid pIS287.

Example 3

Resistance of the above recombinant plasmids to doxorubicin: The level of resistance to daunorubicin and doxorubicin of *S. lividans* TK23 transformed with the recombinant plasmids pIS70, pIS284 or pIS287 in comparison with *S. lividans* TK23, *S. lividans* TK23 transformed with the vector pWHM3 and the anthracycline producing *S. peucetius* ATCC 29050 and ATCC 27952 strains is determined as MICs on R2YE

medium following the procedure described in Materials and Methods. The maximum level of daunorubicin and doxorubicin resistance is obtained with the plasmid pIS287 containing the *drrA, drrB* and *drrC* resistance genes. The level of doxorubicin resistance was increased 64 times also with the plasmid containing only the *drrA* and *drrB*.resistance genes (Table 1).

Table 1. Resistance of recombinant strains to doxorubicin.

Strain	MIC for doxorubicin (micrograms/ml)
S. peucetius ATCC 29050	12.5
S. peucetius ATCC 27952	12.5
S. lividans TK23	12.5
S. lividans TK23(pWHM3)	12.5
S. lividans TK23(pIS284)	800
S. lividans TK23(pIS287)	>800

Example 4

Bioconversion of added daunorubicin to doxorubicin in *S. lividans* TK23 transformed with plasmids containing the *doxA* daunorubicin C-14 hydroxylase gene together with different resistance genes: The pIS70, pIS284 or pIS287 plasmids are introduced into *S. l ividans* TK23 by transformation with selection for thiostrepton resistance, according to the procedures described in the Materials and Methods section. The resulting *S. lividans* TK23(pIS70), *S. lividans* TK23(pIS284) and *S. lividans* TK23(pIS287) transformants are tested for the ability to bioconvert a high level (400 micrograms/ml) of daunorubicin to doxorubicin using the APM medium as described above. *S. lividans* TK23(pIS70) transformants can convert up to 11.5% of added daunorubicin to doxorubicin (Table 2). *S. lividans* TK23(pIS284) and *S. lividans* TK23(pIS287) transformants can convert up to 73.5% of added daunorubicin to doxorubicin (Table 2).

Table 2. Bioconversion of daunorubicin to doxorubicin by *S. lividans* strains.

Strain	Anthracycline (micrograms/ml)		
	DOX	DNR	13-dihydroDNR
S. lividans TK23(pIS70) *(control)*	46	250	70
S. lividans TK23(pIS284)	294	33	21
S. lividans TK23(pIS287)	288	24	35

Example 5

Doxorubicin production in the *S. peucetius* WMH1654 *dnrX* mutant transformed with plasmids containing the *doxA* daunorubicin C-14 hydroxylase gene together with different resistance genes: The pIS284 and pIS287 plasmids are introduced into *S. peucetius* WMH1654 *dnrX* mutant strain by protoplasts transformation with selection for thiostrepton resistance, according to the procedures described in the Materials and Methods section. The resulting *S. peucetius* transformants are fermented and the fermentation broths analyzed according to the method previously described. *S. peucetius* WMH1654(pIS284) produced up to 81 micrograms/ml of doxorubicin and up to 18 micrograms/ml of daunorubicin after a 120 hr fermentation (Table 3). *S.peucetius* WMH1654(pIS287) produced up to 92 micrograms/ml of doxorubicin and no detectable amount of daunorubicin (Table 3).

Table 3. Doxorubicin production by *S. peucetius* WMH1654 *dnrX* strains.

Strain	Anthracycline (micrograms/ml)		
	DOX	DNR	13-dihydroDNR
S. peucetius WMH1654	41	35	18
S. peucetius WMH1654(pIS284)	81	18	6
S. peucetius WMH1654(pIS287)	92	0	0

SEQ ID.1

1 GGATCCGCAC CGGGTACACG GCACGGGACC GCCCACCGCG CGGTGCGCGG

51 TGGGCGGTCC CGTGCCGGTC GCGGCCGGCG GATCAGCGCA GCCAGACGGG

101 CAGTTCGGTG AGCCGCGCCG TCTGGGCCCC CTTCCGGCAC CACCGCAACT

151 CGTCGTACGG CACGGCCAGT CGGGCCTCGG GGAACCTGCT GCGCAGTACG

201 CCGATCATCG TGCGCGACTC CAGCTGGGCG AGCTGCTCCC CGATGCAGTA

251 GTGCGGCCCG TCGCCGAAGG TGAGCCGCCG CCACGAGGGA CGGTCCGGGT

301 GGAAGGCGTG CGGGGCGTCG TGATGGCGGC CGTCGGTGTT GGTGCCCTCG

351 ATGTCCACCA GCACCGGCGC TCCGCGGGGC AGCCGGACGC CGCCGATGGT

401 CACCTCCGTG GCAGCGAACC TCCACAACGT GTAGGGCACC GGCGGGTGGT

451 AGCGCAGCGC CTCCTCCACG AACCGGGAGA CGGCGTCCTC GTCGGCATCC

501 GCCGCGAGGC GGCCCGCCAG GACCTCCGCG AGCAGGAAGC CCAGGAAGGA

551 GCCGGTGGTG TCGTGGCCGG CGAAGATGAG CCCGGTGATC ATGTAGACGA

601 GCTGGTCGTC GGAGACCGAG CCGAACTCGG CCTGCGCGCG CTCGTACAGC

651 ACGCGGGTCA TGGTCGGGGT GTCGTTCCGC CGGGCTGAGT GCACGGCTTC

701 GAGGAGCAGG CTCTCCAGGG CCGAGGTGTC CGGCACGCCC CCGGCAGGGT

751 CCGTGCCGTC ACCCCCGCCG CTCTGCGGGC CGCCGAGGCC GAGTGCCTTG

801 AGAACGCTGA CGGCCTCGCG GGCCATCGCC GGATCGGTGA CCGGCACACC

851 GAGCAGCTCG CAGATGACCA ACAGCGGGAA GTGGTACGCG AAGCCGCCGA

901 TCAGCTCGGC CGGTTTGCCC GACCGGCCGG AGGCGTCGGC GAGTTCGGTG

951 AGCAGCCGGC CGGCGATCGC GGCGATGCGA TCCGTCCGCT CGGCCAGCCG

1001 GCGCGGGTTG AACGCAGGTG CGTGGATGCG GCGCAGGCGC CGGTGGGCCT

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2001 CGACCGCGGC GACCAGCTTC CCGTCGGAGC TGAACGTCGT GTATCCCCCG

2051 GCGCCCGGGT CGCTGACCAC GGTGGCGGTC CAGCCGAACA GGCCGGTGTA

2101 GAAGTCGGCC GAGGCGGCGA CATCGGGCGA ACCGAGGTCG AACCATGCGG

2151 GGGCGCCGGG CGCGAACCTG GTCACGAATC GTTCCTTTCG ATGGATCGGC

2201 ACACGAGCGT CTGCGCTCGC GGATGAGACG GACATCTCGC GGATGAGACG

2251 GACATGCGGG CGGGGCGGGC CGCCGCCGTC AGTGCGCGGT GTCGCCGACG

2301 GCGGCCGCGC CGGCCTCCCA GAGCTTCGCC GCGAGGCCGG CGTCGGCGGT

2351 CGGGCCGCTC ACCGGGGACA GCCGCCGGTC GCTGTAGTAG CCGCCCGTGG

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2451 TTCGCCGCGG AGCGCAGGAA GGGGTTGAAC CGGAAGTAGG ACGAGGCGAC

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2551 CCAGCACGTC CGGCCAGCGC CTGGCGGCCT CCGCCGCGGT CATGATGTTG

2601 GCCTGTTTGG ACGTGCCGTA CGCCTGGCCG GCGCTGTAGC GGTGACGGTC

2651 GCCGTTGAGG TCGTCCGGGT CGATCCGGCC CTGGGTGTAC GCGTCGGACG

2701 AGGTGAGGAT CAGCCGCCCG CCCGCGAGCC GCTCCCGCAG CAGCCGTGCC

2751 AGCAGGAAGC CTGCGAGGTG ATTGACCTGG ATGGTGGCCT CGAACCCGTC

2801 CTGGGTCGTG GTGCGCGACC AGAACATGCC GCCGGCGTTG CTGGCCATGA

2851 CATCGATGCG CGGGTACCGG

CLAIMS

1. A DNA molecule comprising a DNA region containing a gene *dox*A encoding daunorubicin 14-hydroxylase and a DNA region containing at least one gene conferring daunorubicin and doxorubicin resistance.

2. A DNA molecule according to claim 1, further comprising a strong promoter.

3. A DNA molecule according to claim 2, wherein said strong promoter is *erm*E*.

4. A DNA molecule according to claim 1, wherein said gene conferring daunorubicin and doxorubicin resistance is selected from the group consisting of *drr*A, *drr*B and *drr*C genes and any mixtures thereof.

5. A DNA molecule according to claim 4, wherein said genes conferring daunorubicin and doxorubicin resistance are *drr*A and *drr*B genes.

6. The DNA molecule according to claim 4, wherein said genes conferring daunorubicin and doxorubicin resistance are *drr*A, *drr*B and *drr*C genes.

7. The DNA molecule according to claim 1, wherein the region containing the gene *dox*A encoding daunorubicin 14-hydroxylase is 2.9 kb in length.

8. The DNA molecule according to claim 7, wherein the fragment containing the gene *dox*A corresponds to the *Kpn*I-*Bam*HI fragment containing the *dox*A nucleotide sequence.

9. The DNA molecule according to claim 5, wherein said region containing said

*drr*A and *drr*B genes is a 2.3 kb *Xba*I-*Hind*III DNA fragment.

10. The DNA molecule according to claim 1, wherein said genes conferring daunorubicin and doxorubicin resistance are at least 80% identical to genes selected from the group consisting of *drr*A, *drr*B and *drr*C genes.

11. A vector containing a DNA molecule according to claim 1.

12. A vector according to claim 11 wherein said vector is a plasmid.

13. A plasmid according to claim 12, wherein said plasmid is selected from the group consisting of pIS284 and pIS287.

14. A host cell transformed or transfected with a vector according to claim 11.

15. The host cell according to claim 14, wherein said host cell does not produce daunorubicin.

16. The host cell according to claim 14, wherein said host cell is a bacterial cell which produces daunorubicin.

17. The recombinant host cell according to claim 14, wherein said host cell is a Streptomyces cell .

18. A process for bioconverting daunorubicin into doxorubicin, comprising the steps of:

culturing a recombinant host cell in a culture medium containing daunorubicin, wherein said host cell contains a DNA molecule comprising a DNA

region containing a gene *dox*A encoding daunorubicin 14-hydroxylase and a DNA region containing at least one gene conferring daunorubicin and doxorubicin resistance, wherein said host cell does not produce daunorubicin, and

isolating any resulting doxorubicin from the culture medium.

19. A process for producing doxorubicin by fermentation, comprising the steps of:

culturing a recombinant host cell in a culture medium, wherein said host cell contains a DNA molecule comprising a DNA region containing a gene *dox*A encoding daunorubicin 14-hydroxylase and a DNA region containing one or more genes conferring daunorubicin and doxorubicin resistance, wherein said host cell is a bacterial cell which produces daunorubicin, and

isolating any resulting doxorubicin from the culture medium.

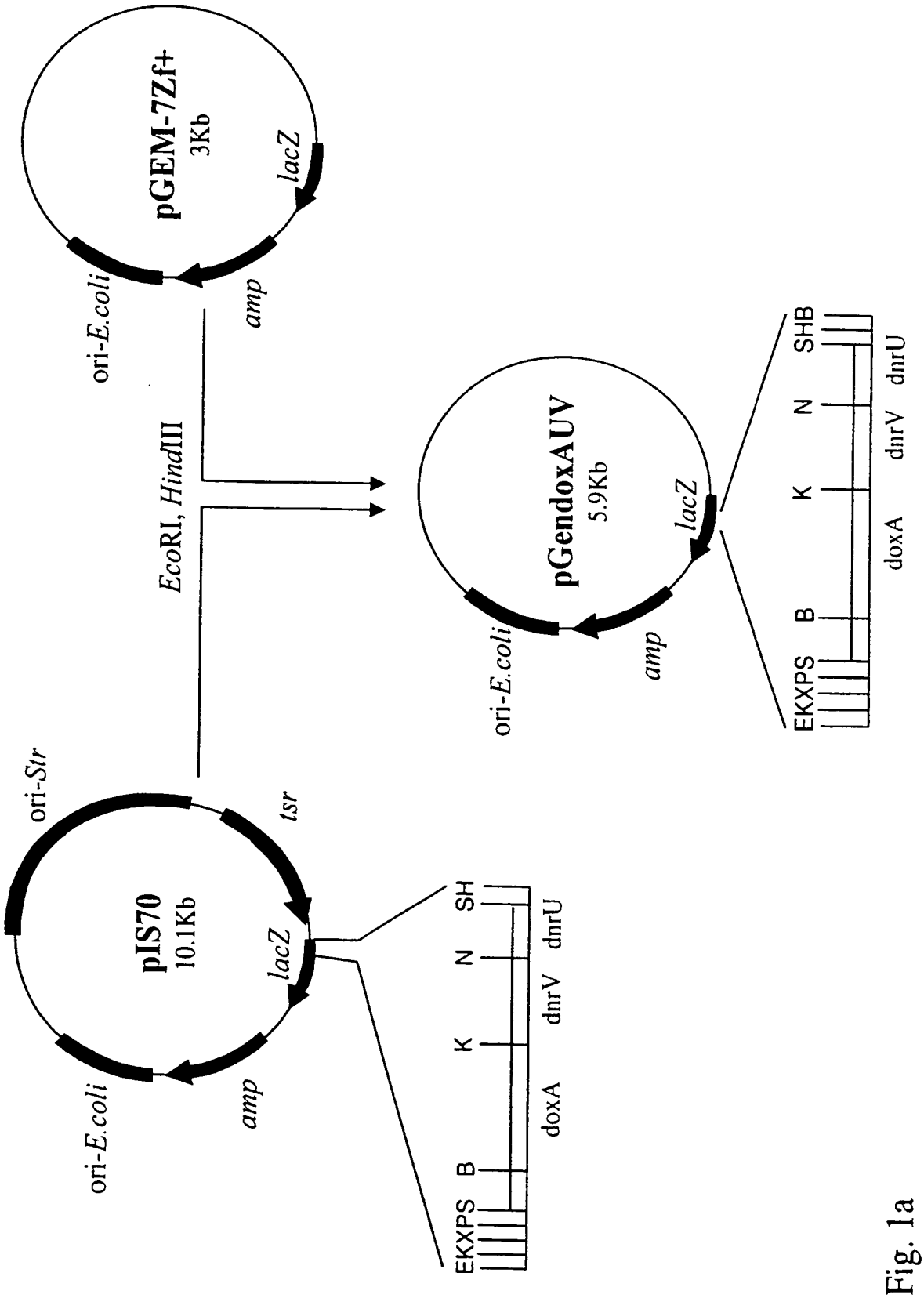
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Fig. 1a

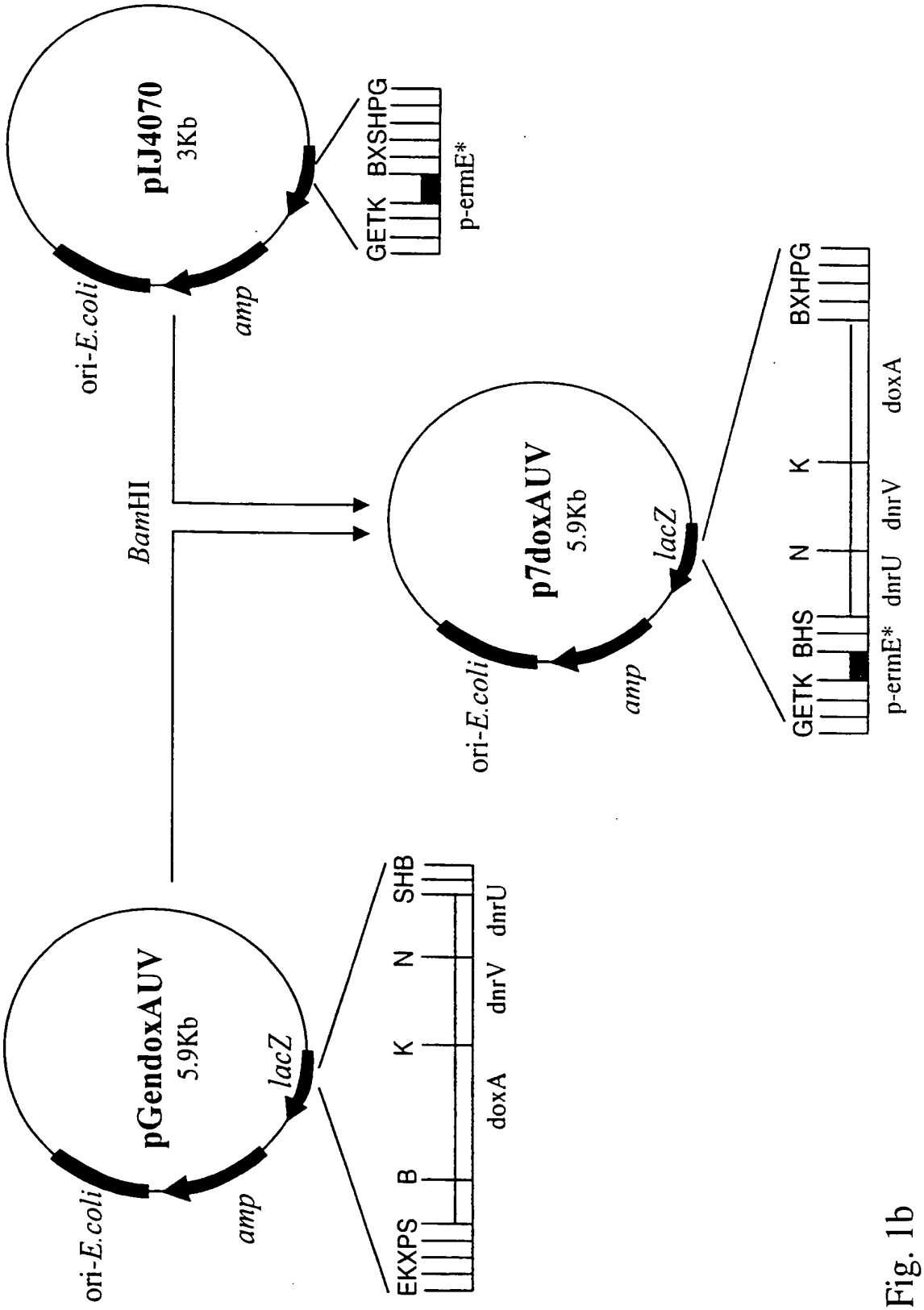
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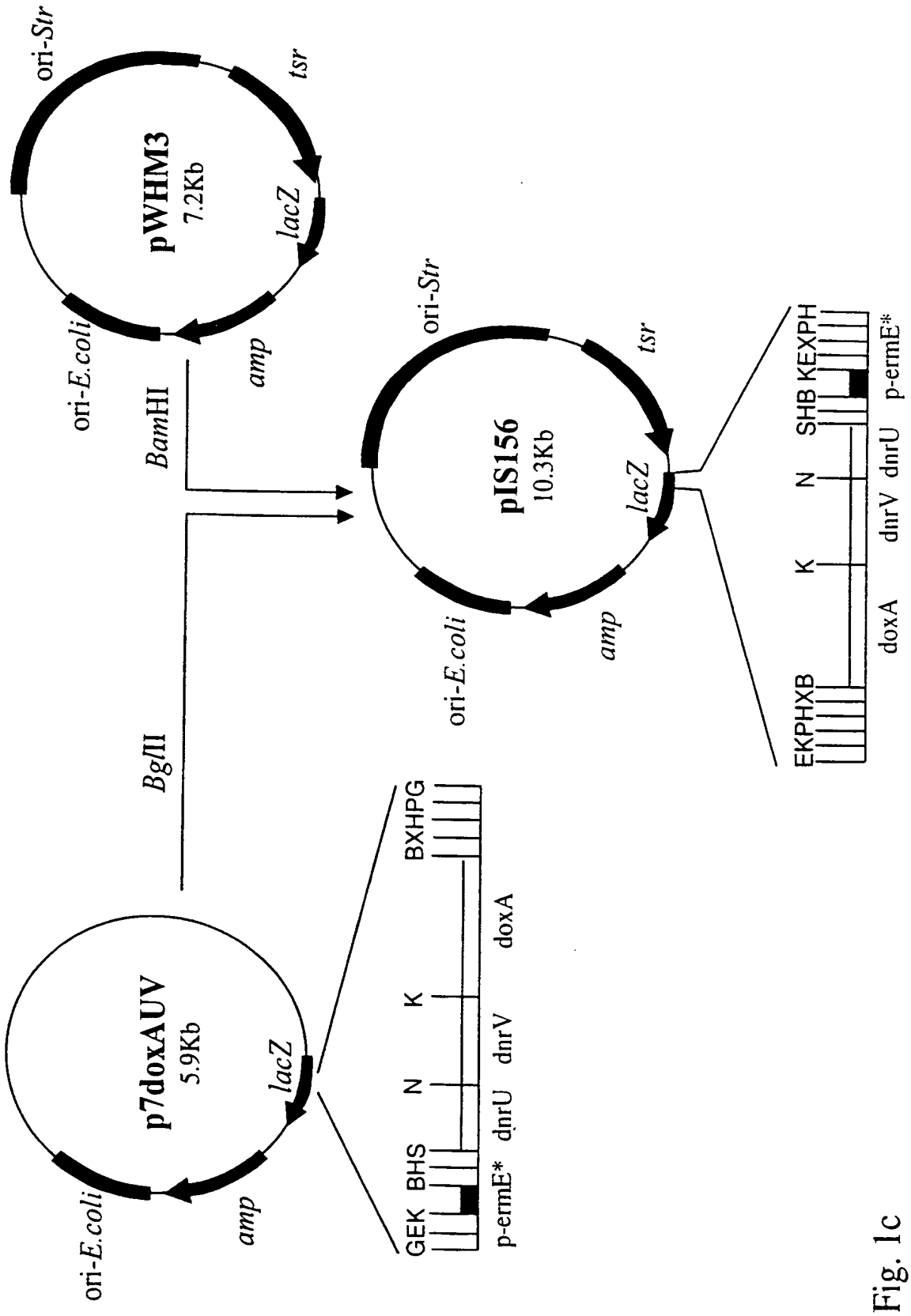
Fig. 1b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lacZ
ori-E.coli
amp
p7doxAUV
5.9Kb
lacZ
ori-E.coli
amp
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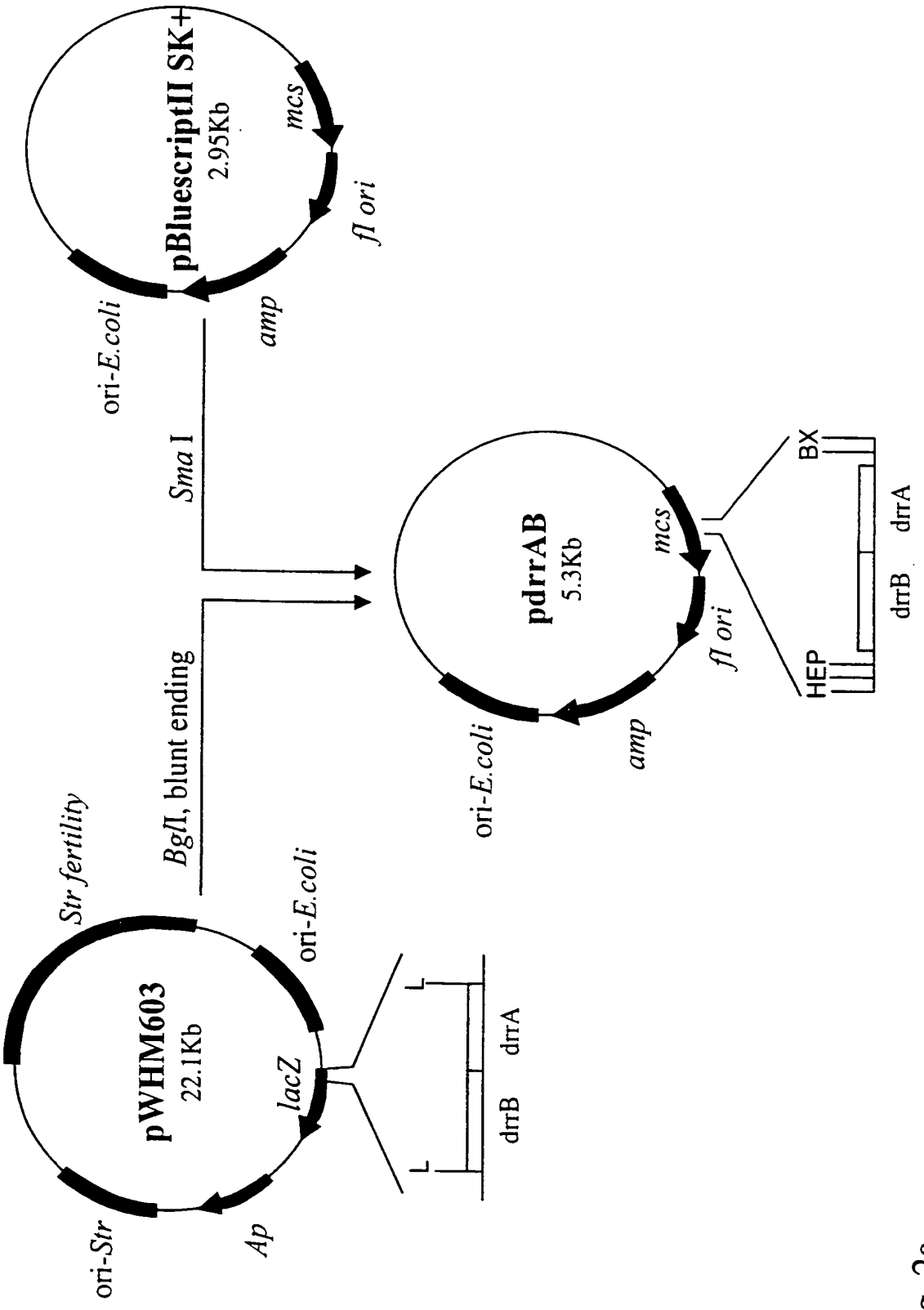
Fig. 1c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ori-E.coli
BX
HEP
drrB
drrA
BglI, blunt ending
Str fertility
pWHM603
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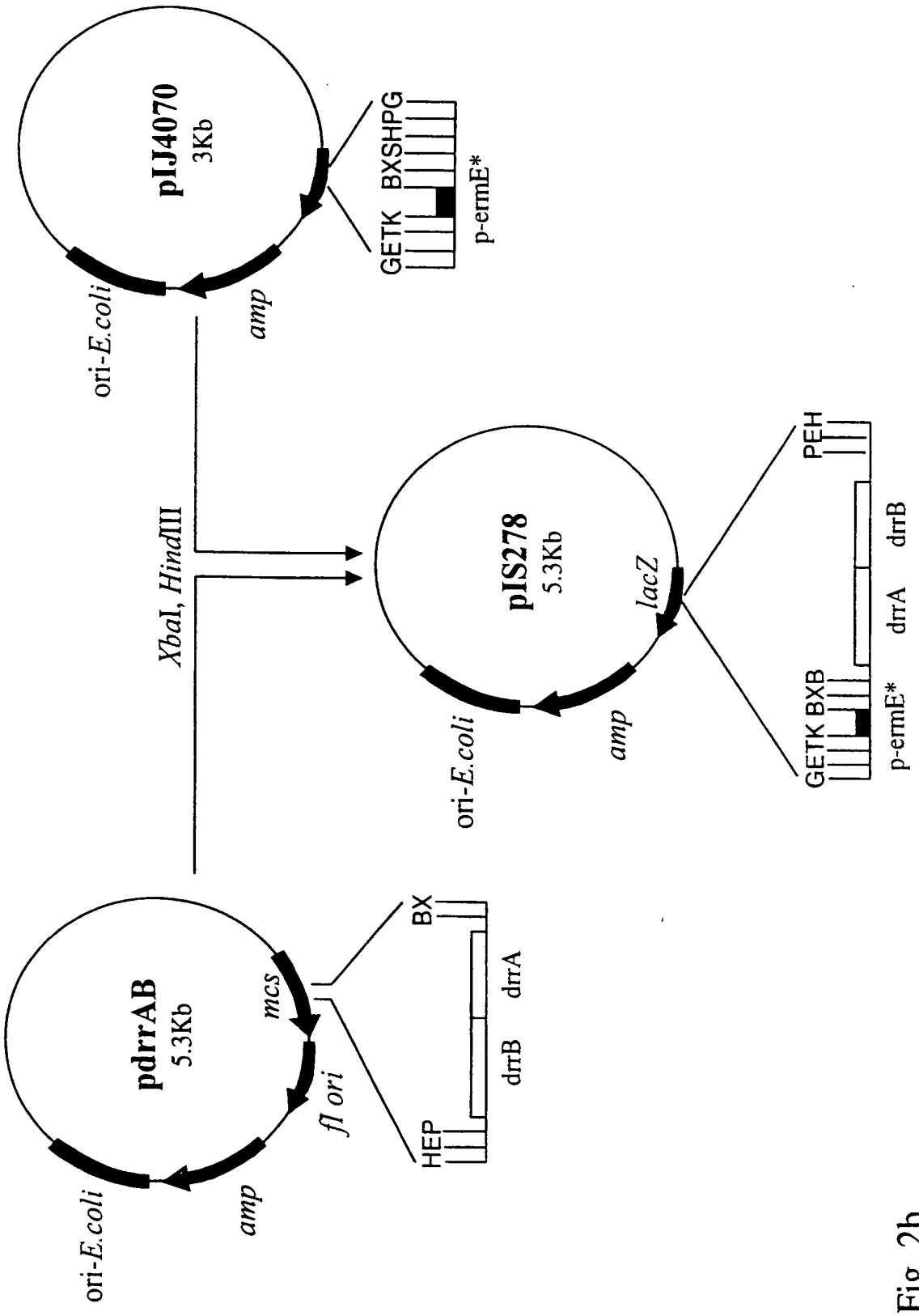
lacZ
ori-E.coli
ori-Str
Ap
L
L
drrB
drrA

Fig. 2a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5.3Kb
ori-E.coli
amp
lacZ
PEH
GETK BXB
p-ermE*
drrA
drrB
pdrrAB
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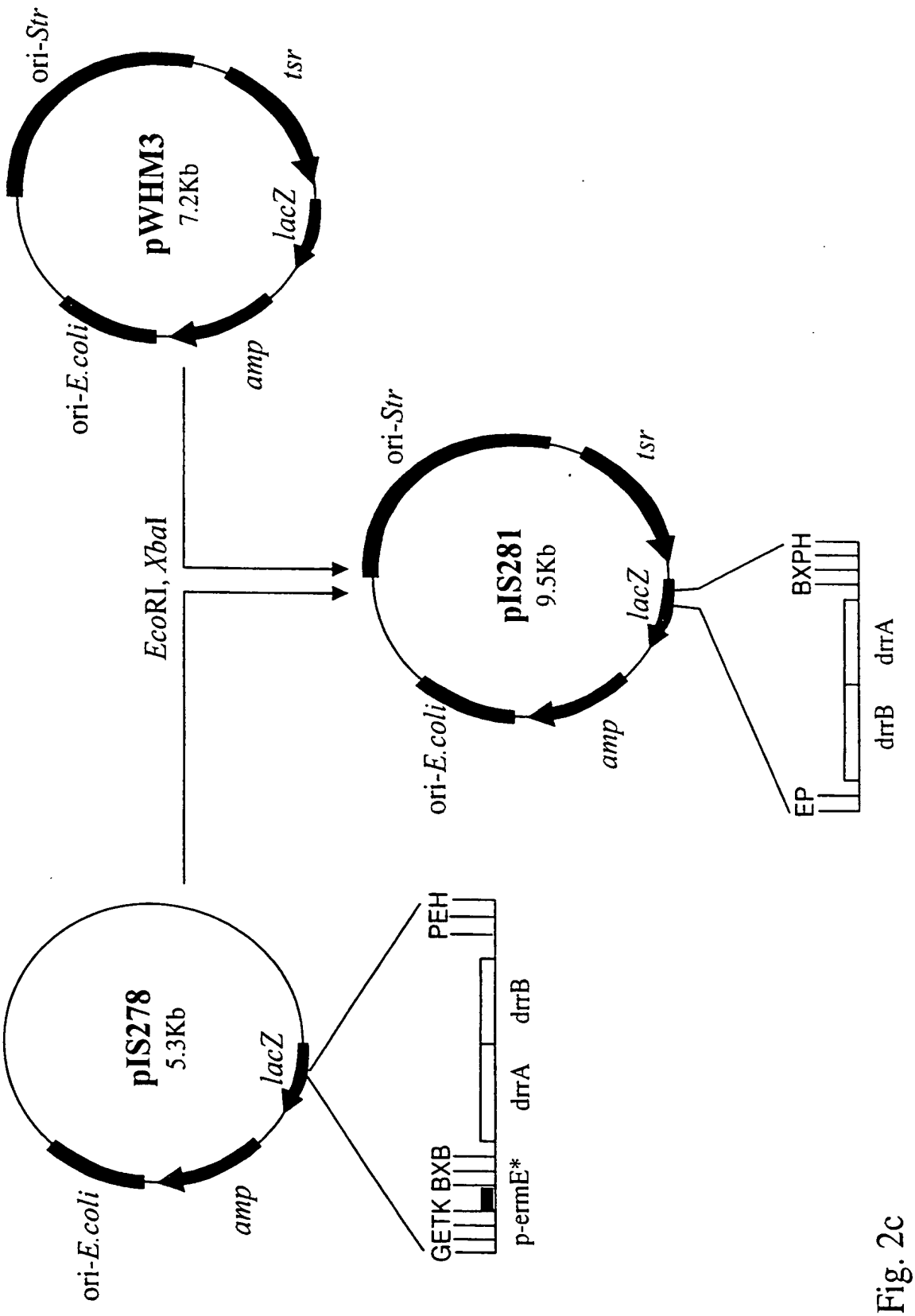
amp
fl ori
mcs
HEP
drrB
BX
drrA

Fig. 2b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lacZ
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ori-Str
tsr
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9.5Kb
lacZ
amp
ori-E.coli
BXPH
drrA
drrB
EP
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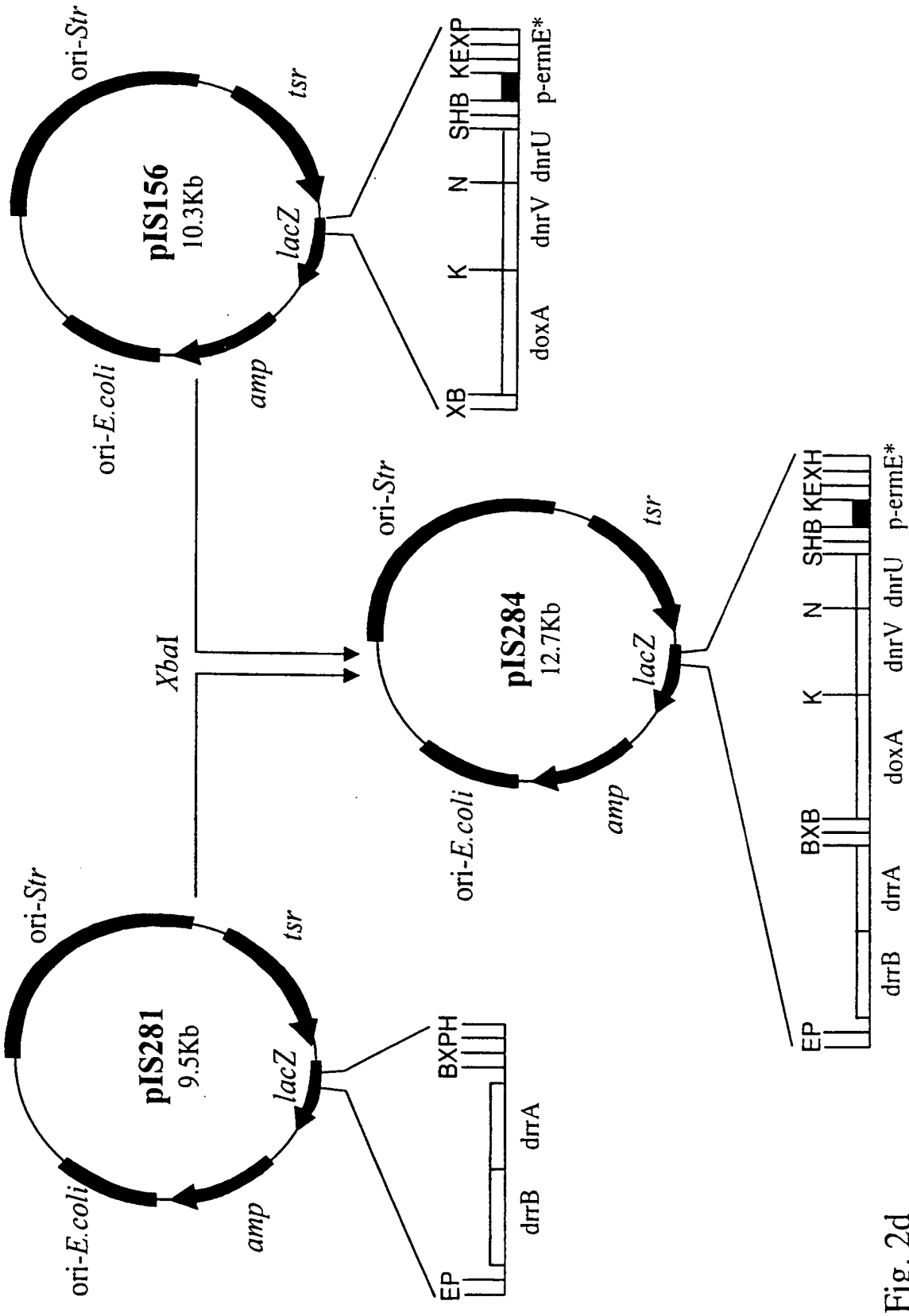
lacZ
amp
ori-E.coli
PEH
drrB
drrA
GETK BXB
p-ermE*

Fig. 2c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SHB
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doxA
dnrV
dnrU
p-ermE*
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EP
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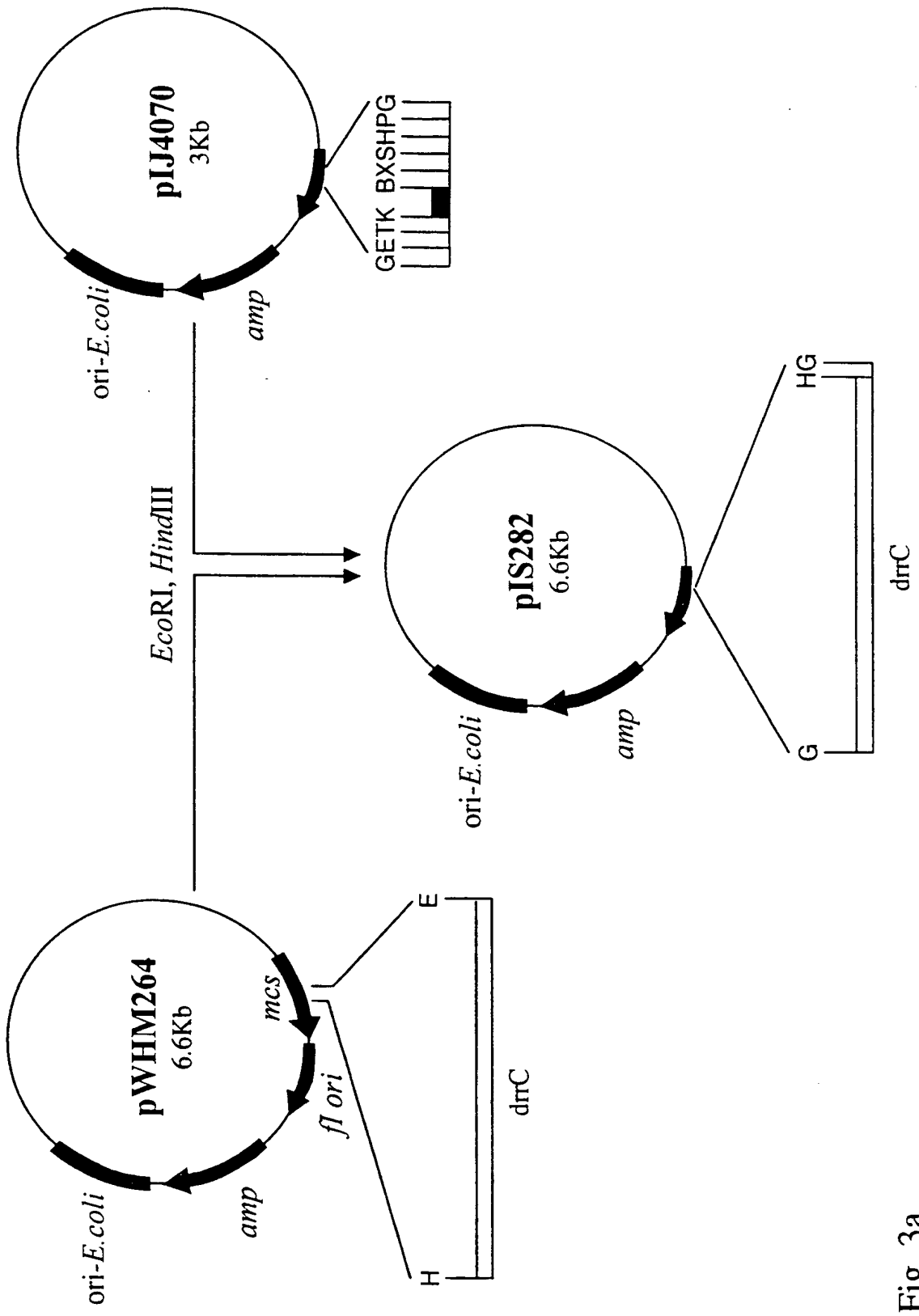
pIS281
9.5Kb
BXPH

Fig. 2d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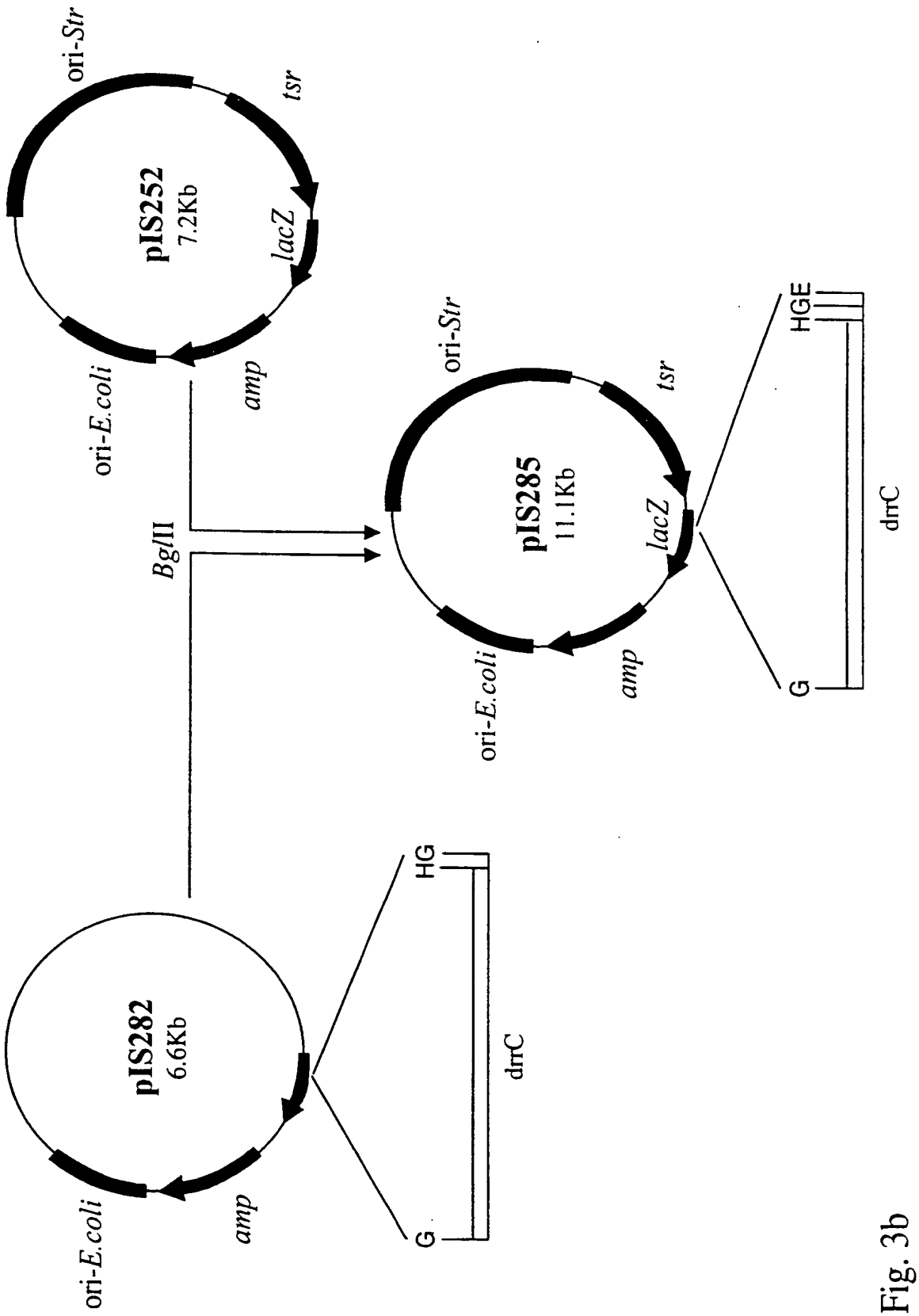
mcs
f1 ori
E
H
drrC
ori-E.coli
amp

Fig. 3a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pIS285
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lacZ
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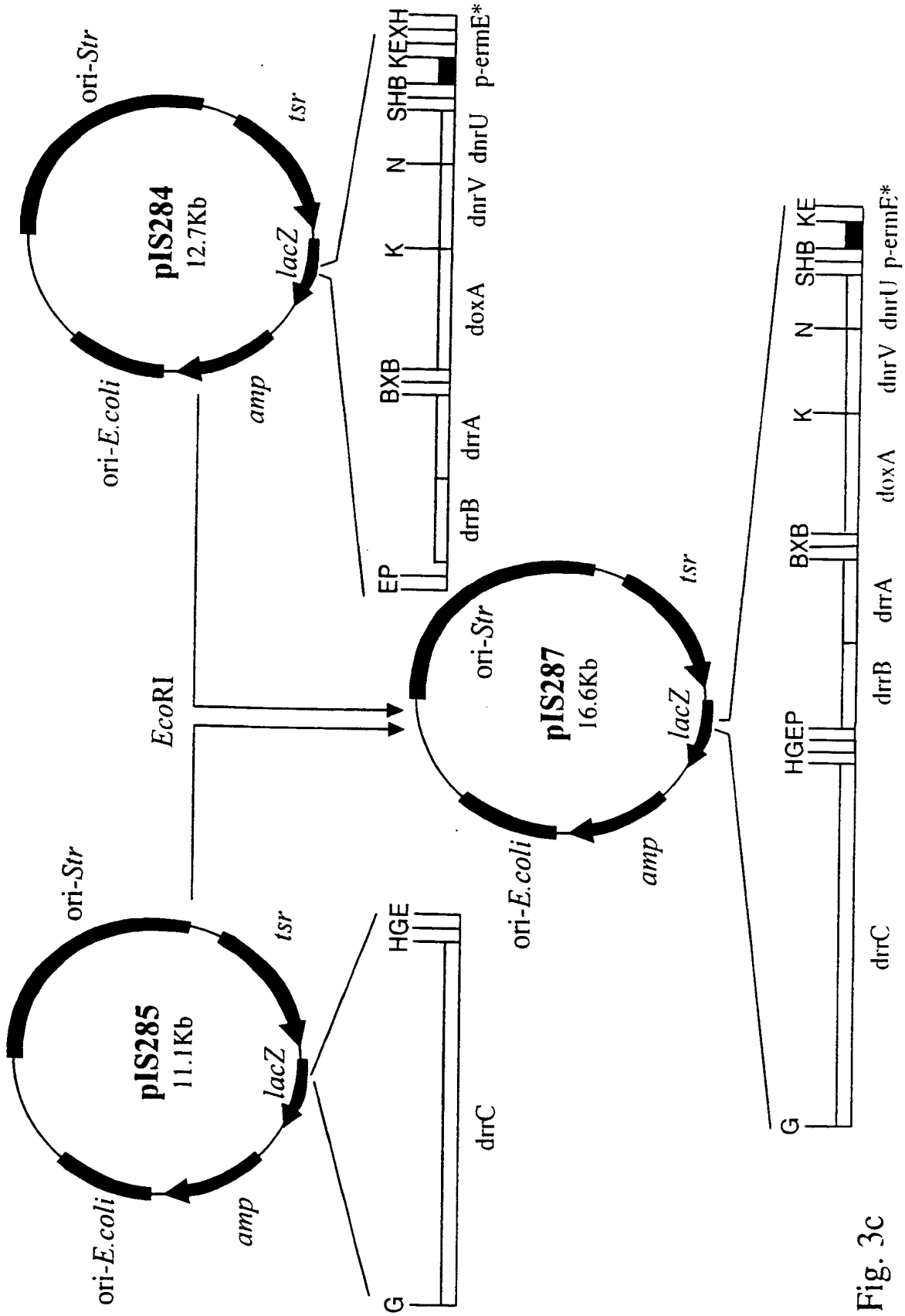
G
drrC
pIS282
6.6Kb
ori-E.coli
amp
HG
G
drrC

Fig. 3b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N
K
dnrV dnrU p-ermE*
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doxA
drrA
drrB
EP
EcoRI
ori-Str
tsr
pIS287
16.6Kb
lacZ
ori-E.coli
amp
BXB
K
SHB KE
N
dnrV dnrU p-ermE*
doxA
drrA
drrB
HGEP
drrC
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lacZ
ori-E.coli
amp
HGE
drrC
G

Fig. 3c

SEQUENCE LISTING

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<130> 1615-9003

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<141> 1999-04-22

<150> 09/065,606
<151> 1998-04-24

<160> 1

<170> PatentIn Ver. 2.0

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<213> Streptomyces peucetius

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<222> Complement((1)..(2870))
<223> Complementary strand to the coding strand

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tctgggcccc cttccggcac caccgcaact cgtcgtacgg cacggccagt cgggcctcgg 180
ggaacctgct gcgcagtacg ccgatcatcg tgcgcgactc cagctgggcg agctgctccc 240
cgatgcagta gtgcggcccg tcgccgaagg tgagccgccg ccacgaggga cggtccgggt 300
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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Articl 18 and Rules 43 and 44)

Applicant's or agent's file reference F1615-9003	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US99/07016	International filing date *(day/month/year)* 22 APRIL 1999	(Earliest) Priority Date *(day/month/year)* 24 APRIL 1998
Applicant PHARMACIA & UPJOHN S.P.A.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [ ] **Unity of invention is lacking** (See Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [X] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] transcribed by this Authority.

4. With regard to the title,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. 1a
   - [X] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07016

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 19/56; C12N 15/31, 15/53, 15/63, 15/74, 15/76
US CL :435/78, 252.3, 252.35, 320.1; 536/23.2, 23.7

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/78, 252.3, 252.35, 320.1; 536/23.2, 23.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,695,966 A (INVENTI et al) 09 December 1997, columns 3-8, Figures 1 and 2, and SEQ IDs NOs:1 & 2.	1-19
Y	DICKENS, M. L. Isolation and Characterization of a Gene from *Streptomyces* sp. Strain C5 That Confers the Ability To Convert Daunomycin to Doxorubicin on *Streptomyces lividans* TK24. Journal of Bacteriology. June 1996, Vol. 178, No. 11, pages 3389-3395, especially pages 3390-3394 and Figures 2 and 3.	1-7, 9-12, and 14-19
Y	WO 97/44439 A2 (THE OHIO STATE UNIVERSITY RESEARCH FOUNDATION) 27 November 1997, pages 6-28, Figures 2 and 3 and SEQ IDs NOs:4 and 5.	1-7, 9-12, and 14-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
07 SEPTEMBER 1999

Date of mailing of the international search report
25 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
WILLIAM W. MOORE
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07016

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HWANG, K. H. et al. Expression of *Streptomyces peucetius* Genes for Doxorubicin Resistance and Aklavinone 11-Hydroxylase in *Streptomyces galilaeus* ATCC 31133 and Production of a Hybrid Aclacinomycin. Antimicrobial Agents and Chemotherapy. July 1995, Vol. 39, No. 7, pages 1616-1620, especially pages 1617-1619.	1, 2, and 4-19
Y	KAUR, P. Expression and Characterization of DrrA and DrrB Proteins of *Streptomyces peucetius* in *Escherichia coli*: DrrA Is an ATP Binding Protein. Journal of Bacteriology. February 1997, Vol. 179, No. 3, pages 569-575, especially pages 570-574 and Figures 3 and 4.	1, 2, and 4-19
Y	LOMOVSKAYA. N. et al. *The Streptomyces peucetius drrC* Gene Encodes a UvrA-Like Protein Involved in Daunorubicin Resistance and Production. Journal of Bacteriology. June 1996, Vol. 178, No. 11, pages 3238-3245, especially pages 3240-3244 and Figure 2.	4, 6, 10 and 13
Y	WO 97/06266 A1 (ABBOTT LABORATORIES) 20 February 1997, pages 11-13, 15 and 16.	2, 3 and 13
A	US 5,364,781 A (HUTCHINSON et al) 15 November 1994, columns 3-10.	1, 2, 11, 12, 14-17 and 19
A	US 5,652,125 A (SCOTTI et al) 29 July 1997, columns 3-10.	1, 2, 11, 12, 14-17 and 19
A	US 5,665,564 A (CARUSO et al) 09 September 1997, columns 1-6.	1, 4-6 9, and 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07016

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

SEQ ID NO:1, as nucleotide sequence and translated amino acid sequence in N-GeneSeq36, GenEMBL(various), issued U.S. application nucleotide sequences, A-GeneSeq36, PIR60, SwissProt37, SPTREMBL10, issued U.S. application amino acid sequences; STN/Chemical Abstracts; DIALOG/Medline, Biosis, Agricola, Current BioTechnology Abstracts, Derwent Biotechnology Abstracts

5

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rul 70)

Applicant's or agent's file reference P1615-9003	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/07016	International filing date *(day/month/year)* 22 APRIL 1999	Priority date *(day/month/year)* 24 APRIL 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant PHARMACIA & UPJOHN S.P.A.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05 NOVEMBER 1999	16 JUNE 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PONNATHAPURA ACHUTAMURTHY Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/07016

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the internati nal application as riginally filed

[X] the description:
pages 1-19 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 20-22 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-10 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages 1-2 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/07016

V. Reasoned statement under Article 35(2) with regard to novelty, inventiv step r industrial applicability; citations and explanations supporting such statement

1. stat ment

Novelty (N)	Claims	1-19	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-19	NO
Industrial Applicability (IA)	Claims	1-19	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-5 and 7-19 lack an inventive step under PCT Article 33(3) as being obvious over Inventi et al., U.S. Patent No. 5,695,966, and either WO 97/44439 or the corresponding article by Dickens et al. in view of the published International applications and WO 89/11532 and WO 97/06266. The more recent publications of Hwang et al. and Kaur are also cited herein because they corroborate the disclosures of WO 89/11532.

Each of Inventi et al.('966), WO 97/44439, and a corresponding article by Dickens et al., was cited in the Search Report and each disclose the identification and isolation of genomic DNA regions comprising *dox*A genes encoding the daunomycin[doxorubicin] C-14 hydroxylases of, respectively, *Streptomyces peucetius* 29050 and *Streptomyces spp.* strain C5 corresponding to limitations of **claim 1** herein. Dickens et al. is discussed rather than WO 97/44439. Both Inventi et al. ('966) and Dickens et al. further disclose that they placed the *dox*A genes in plasmid expression vectors in operable linkage with strong promoters corresponding to limitations of **claim 2** herein and transformed *Streptomyces* cells that either produce or do not produce daunorubicin, yet incapable of producing doxorubicin, according to **claims 14-17** herein, in order to conduct processes meeting the limitations of **claims 18 and 19** herein. See Examples 1 and 2 of Inventi et al. and pages 3391-3394 of Dickens et al. Both Inventi et al.('966) and Dickens et al. also disclose the characterization of these *dox*A genes encoding cytochrome P450-like polyketide hydroxylases that convert daunomycin[daunorubicin] to doxorubicin. Inventi et al. more particularly disclose, in Fig. 1, the 2.9kb *Bam*HI-*Sph*I restriction endonuclease segment of **claims 7 and 8** herein which comprises the internal *dox*A 1269-nucleotide coding sequence, while Dickens et al. disclose an internal 1269-nucleotide *dox*A coding sequence specifying a nearly identical DoxA amino acid sequence.

Neither Inventi et al. nor WO 97/44439 and Dickens et al. disclose the use of the *Saccharopolyspora erythraea erm*E* promoter in an expression plasmid, but WO 97/06266 discloses this required element of **claims 2, 3 and 13** herein in preparation of expression plasmids - see Examples 3, 5, 7 and 8 at pages 11-16 - in order to promote the high-level expression of polyketide hydroxylases in actinomycetes generally, including *Streptomyces* species. Thus it (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation f: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C12P 19/56; C12N 15/31, 15/53, 15/63, 15/74, 15/76 and US Cl.: 435/78, 252.3, 252.35, 320.1; 536/23.2, 23.7

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
would have been obvious to one of ordinary skill in the art at the time the invention is made to have substituted the *ermE** promoter disclosed by WO 97/44439 for a strong promoter used by Inventi et al. or Dickens et al. in an expression plasmid comprising a polyketide hydroxylase-encoding gene. This is because WO 97/44439 teaches that this promoter may be operably-linked in an expression plasmid to a gene encoding a polyketide hydroxylase wherein the plasmid is suitable for gene expression in *Streptomyces* species whereby it is advantageous for driving high-level expression of the hydroxylase product.

While none among Inventi et al. ('966), WO 97/44439, Dickens et al., nor WO 97/06266 disclose the identification or the isolation of any doxorubicin resistance genes or the use of such genes in a transformed host cell in a process for producing doxorubicin, each of the disclosures of WO 89/11532, Hwang et al. and Kaur describes the identification, the isolation, and the use of plasmids comprising such resistance genes, the *drr*A and *drr*B genes, to confer a doxorubicin-resistant phenotype on *Streptomyces* host cells capable of producing doxorubicin transformed with the plasmids, thereby permitting increased doxorubicin production by a transformed host cell. See, Figures 1 and 2 and the accompanying disclosure at pages 8-13 and of WO 89/11532, inherently disclosing the *Xba*I-*Hin*dIII restriction endonuclease segment required by **claim 9** herein. See also, Figures 1-4, 6 and 7 and the accompanying disclosure at pages 572-575 of Kaur. See further, Figures 2 and 3, Table I, and the disclosure at pages 1616-1619 of Hwang et al. It would have been obvious to one of ordinary skill in the art at the time the invention was made to prepare a DNA molecule comprising a *dox*A gene disclosed by Inventi et al. or by WO 97/44439 and Dickens et al. and further comprising either or both of the *drr*A and *drr*B genes according to limitations of **claims 1-5** herein, and to prepare plasmids and the transformed *Streptomyces* host cells maintaining the plasmids according to **claims 10-17** herein as well in order to conduct processes of **claims 18 and 19** herein. This is because the prior art teaches that increased production of doxorubicin results in increased toxicity of the product for a producing host cell and that such toxicity may be relieved by instituting or augmenting doxorubicin resistance, a phenotype conferred by both the products of the *drr*A and *drr*B genes.

Claims 6 and 13 lack an inventive step under PCT Article 33(3) as being obvious over the prior art as applied in the immediately preceding paragraph and further in view of Lomovskaya et al. who teaches the identification and isolation of a gene encoding the *drr*C gene which also confers resistance to doxorubicin. It would have been obvious to one of ordinary skill in the art at the time the invention was made to have incorporated the drrC gene taught by Lomovskaya et al. in a DNA molecule according to **claim 6** herein and to further prepare an expression plasmid of **claim 13** herein in order to transform a *Streptomyces* host cell to conduct processes of claims 18 and 19 herein because the prior art in general teaches that increased doxorubicin production results in increased toxicity of the product for a producing host cell and that such toxicity may be relieved by instituting or augmenting doxorubicin resistance, a phenotype conferred by the product of the *drr*C gene.

Claims 1-19 meet the criteria set out in PCT Articles 33(2) and 33(4), because no single prior art publication simultaneously describes all of the elements of the subject matter of any single claim herein and because the subject matter of each of claims 1-19 is enabled by the present disclosure and is industrially applicable.

---------- NEW CITATIONS ----------
NONE

PATENT COOPERATION TREATY

From the RECEIVING OFFICE

To:

ROBERT B. MURRAY
NIKAIDO, MARMELSTEIN, MURRAY & ORAM, LLP
METROPOLITAN SQUARE, SUITE 330-G STREET
LOBBY, 655 15TH STREET, N.W.
WASHINGTON DC 20005-5701

PCT

NOTIFICATION OF THE INTERNATIONAL APPLICATION NUMBER AND OF THE INTERNATIONAL FILING DATE

(PCT Rule 20.5(c))

Date of mailing (*day/month/year*): 20 MAY 1999

Applicant's or agent's file reference: F1615-9003

IMPORTANT NOTIFICATION

International application No.	International filing date (*day/month/year*)	Priority date (*day/month/year*)
PCT/US99/07016	22 APR 99	24 APR 98

Applicant: PHARMACIA & UPJOHN S.P.A.

Title of the invention: PROCESS FOR PREPARING DOXORUBICIN

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.

2. The applicant is further notified that the record copy of the international application:

☑ was transmitted to the International Bureau on 20 MAY 1999.

☐ has not yet been transmitted to the International Bureau for the reason indicated below and a copy of this notification has been sent to the International Bureau*:

☐ because the necessary national security clearance has not yet been obtained.

☐ because (*reason to be specified*):

* The International Bureau monitors the transmittal of the record copy by the receiving Office and will notify the applicant (with Form PCT/IB/301) of its receipt. Should the record copy not have been received by the expiration of 14 months from the priority date, the International Bureau will notify the applicant (Rule 22.1(c)).

3. FOREIGN TRANSMITTAL LICENSE INFORMATION Completed by: DHH

☐ Additional license for foreign transmittal not required. This subject matter is covered by a license already granted on the equivalent U.S. national application. Refer to that license for information concerning its scope.

☐ License for foreign transmittal not required. 37 CFR 5.11(e)(1) or 37 CFR 5.11(e)(2). However, a license may be required for additional subject matter. See 37 CFR 5.15(b).

☑ Foreign transmittal license granted. 35 U.S.C. 184; 37 CFR 5.11 on 5-7-99 (date):

☑ 37 CFR 5.15(a) ☐ 37 CFR 5.15(b)

Name and mailing address of the receiving Office	Authorized officer
Assistant Commissioner for Patents Box PCT Washington, D.C. 20231 Attn: RO/US	Elaine Rivera Paralegal Specialist IAPD - PCT Operations
Facsimile No.	Telephone No. (703) 305-3573

Form PCT/RO/105 (July 1992)

PATENT COOPERATION T TY

From the RECEIVING OFFICE

To:

ROBERT B. MURRAY
NIKAIDO, MARMELSTEIN, MURRAY & ORAM, LLP
METROPOLITAN SQUARE, SUITE 330-G STREET
LOBBY, 655 15TH STREET, N.W.
WASHINGTON DC 20005-5701

PCT

INVITATION TO CORRECT DEFECTS IN THE INTERNATIONAL APPLICATION

(PCT Articles 3(4)(i) and 14(1) and Rule 26)

Date of mailing (*day/month/year*)	20 MAY 1999
Applicant's or agent's file reference: F1615-9003	**REPLY DUE** within **ONE MONTH** from the above date of mailing
International application No.: PCT/US99/07016	International filing date (*day/month/year*): 22 APR 99
Applicant: PHARMACIA & UPJOHN S.P.A.	

1. ☑ The applicant is hereby invited, within the time limit indicated above, to correct, **in the international application as filed**, the defects specified on the attached
   - ☑ Annex A
   - ☐ Annex B1 *(text matter of the international application as filed)*
   - ☐ Annex C1 *(drawings of the international application as filed)*

2. ☐ The applicant is hereby invited, within the time limit indicated above, to correct, **in the translation of the international application** furnished under Rule 12.3, the defects specified on the attached
   - ☐ Annex A
   - ☐ Annex B2 *(text matter of the translation of the international application)*
   - ☐ Annex C2 *(drawings of the translation of the international application)*

Additional observations (if necessary):

HOW TO CORRECT THE DEFECTS?

Correction must be submitted by filing a replacement sheet embodying the correction and a letter accompanying the replacement sheet, which shall draw attention to the difference between the replaced sheet and the replacement sheet. A correction may be stated in a letter only if it is of such a nature that it can be transferred from the letter to the record copy without adversely affecting the clarity and direct reproducibility of the sheet onto which the correction is to be transferred (Rule 26.4).

ATTENTION

Failure to correct the defects will result in the international application being considered withdrawn by this receiving Office (see Rule 26.5 for further details).

A copy of this invitation and any attachments has been sent to the International Bureau
☑ and the International Searching Authority.

Name and mailing address of the receiving Office Assistant Commissioner for Patents Box PCT Washington, D.C. 20231 Attn: RO/US Facsimile No.	Authorized officer Elnora Rivera Paralegal Specialist IAPD - PCT Operations Telephone No. (703) 305-3873

Form PCT/RO/106 (July 1998)

ANNEX A TO FORM PCT/RO/106

International application No.
PCT/US99/07016

The receiving Office has found the following defects in the international application as filed:

1. As to **signature*** of the international application (Rules 4.15 and 90.4), the request:
   a. ☐ is not signed.
   b. ☐ is not signed by all the applicants.
   c. ☐ is not accompanied by the statement referred to in the check list in Box No. VIII of the request explaining the lack of the signature of an applicant for the designation of the United States of America.
   d. ☑ is signed by what appears to be an agent/common representative but
      ☑ the international application is not accompanied by a power of attorney appointing him.
      ☐ the power of attorney accompanying the international application was not signed by all the applicants.
   e. ☐ other *(specify)*:

✔ All applicants must sign, including inventors if they are also applicants (e.g. where the United States of America is designated).

2. As to indications concerning the **applicant**, the request (Rules 4.4 and 4.5):
   a. ☐ does not properly indicate the applicant's name *(specify)*:
   b. ☐ does not indicate the applicant's address.
   c. ☐ does not properly indicate the applicant's address *(specify)*:
   d. ☐ does not indicate the applicant's nationality.
   e. ☐ does not indicate the applicant's residence.
   f. ☐ other *(specify)*:

3. As to the **language** of certain elements of the international application, other than the description and claims (Rule 12.1(c) and 26.3*ter*(a) and (c)):
   a. ☐ **the request** is not in a language which is both a language accepted by this receiving Office and a language of publication, which is (are):
   b. ☐ **the text matter of the drawings** is not in the language in which the international application is to be published, which is:
   c. ☐ **the abstract** is not in the language in which the international application is to be published, which is:

4. The **title** of the invention:
   a. ☐ is not indicated in Box No. I of the request (Rule 4.1(a)).
   b. ☐ is not indicated at the top of the first sheet of the description (Rule 5.1(a)).
   c. ☐ as appearing in Box No. I of the request is not identical with the title heading the description (Rule 5.1(a)).

5. As to the **abstract** (Rule 8):
   ☐ the international application does not contain an abstract.

PATENT COOPERATION TREATY

PCT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: ROBERT B. MURRAY
NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP
METROPOLITAN SQUARE
655 FIFTEENTH STREET N.W.
SUITE 330 - G STREET LOBBY
WASHINGTON, DC 20005-5701

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	21 JUL 2000

Applicant's or agent's file reference	IMPORTANT NOTIFICATION
P1615-9003	

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US99/07016	22 APRIL 1999	24 APRIL 1998

Applicant
PHARMACIA & UPJOHN S.P.A.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	PONNATHAPURA ACHUTAMURTHY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

P. ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 17 December 1999 (17.12.99)	
International application No. PCT/US99/07016	**Applicant's or agent's file reference** F1615-9003
International filing date (day/month/year) 22 April 1999 (22.04.99)	**Priority date** (day/month/year) 24 April 1998 (24.04.98)
Applicant SOLARI, Augusto, Inventi et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

05 November 1999 (05.11.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Jean-Marie McAdams
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

3019749

IN THE INTERNATIONAL BUREAU OF WIPO

In re the application of:
PHARMACIA & UPJOHN S.P.A.
PCT International Application Number: PCT/US99/07016
Filed: 22 April 1999
For: Process for Preparing Doxorubicin

Response to Communication Concerning C-I-P Data Omitted In the PCT Request

International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

15 July 1999

Dear Sir:

This is our response to the Communication dated 17 June 1999 from the International Bureau concerning the date of the continuation-in-part listed on Sheet 4 of the PCT Request form.

The communication indicated that the date of the continuation-in-part on Sheet 4 was missing. The applicants respectfully submit a replacement of the Supplemental Sheet of the Request form to provide the necessary information. The Continuation of Box V furnished on the revised Supplemental Sheet states that the date of the United States application serial number 09/065,606 is 24 April 1998.

It is believed that the issue raised in this communication has been satisfied.

Respectfully submitted,
NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Robert B. Murray

Agent Docket No. F1615-9003
Metropolitan Square
655 Fifteenth Street, N.W.
Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000
RBM/arw

Enclosure: Replacement Sheet 5

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:** in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV:*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication **"patent of addition,"** or **"certificate of addition,"** or if, in Box No. V., the name of the United States of America is accompanied by an indication **"continuation"** or **"continuation-in-part"**: in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI) , the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed:** in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, the **earlier application is an ARIPO application:** in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudical disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation of Box No. IV

NIKAIDO, David T.
MARMELSTEIN, Charles M.
ORAM, George E., Jr.
EMAS, Ellen Marcie
GOLDHUSH, Douglas H.
KITTS, Monica Chin
BERMAN, Richard J.
WONG, King L.
MUIR, Patrick D.
KLESNER, Sharon Nolan

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Metropolitan Square
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Continuation of Box No. V

US: 24 April 1998 (24.04.98)